Association between the C34T polymorphism of the *AMPD1* gene and essential hypertension in Malaysian patients

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ABSTRACT. The aim of this study was to determine whether C34T, a common polymorphism of the adenosine monophosphate deaminase 1 gene (*AMPD1*), is associated with essential hypertension (EH). We

hypothesize that C34T is associated with the development of EH. A case-control design was used for this study. The DNA was extracted using a commercial kit from the whole blood of 200 patients with hypertension and 200 subjects without hypertension from selected Malaysian ethnicities (Malays, Chinese, and Indians). Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) and agarose gel electrophoresis were used for genotyping. The C34T gene polymorphism of *AMPD1* was significantly associated with EH in the Malaysian subjects (P < 0.0001). The genotype frequencies of CC, CT, and TT were 6%, 79%, and 15%, respectively, among hypertensive subjects, while no TT genotypes were observed in the normotensive subjects. Further, the frequency of hypertension was higher among T allele carriers than C carriers (OD = 9.94; 95%CI = 6.851-14.434). There were significant differences in the systolic blood pressure, diastolic blood pressure, and pulse pressure (P < 0.05) between the normotensive and hypertensive Malaysian subjects; we believe those difference were caused by the C34T polymorphism. For the first time in Malaysia, the current study provides evidence that a common polymorphism of the *AMPD1* gene (C34T) is strongly associated with EH.

Key words: Essential hypertension; *AMPD1*; C34T; Polymorphism; Malaysia

INTRODUCTION

Adenosine monophosphate deaminase (AMPD) converts adenosine monophosphate (AMP) to inosine monophosphate (IMP). In cases of AMPD deficiency, there is an inability to convert AMP to IMP. As a result, adenosine is produced (Morisaki et al., 1992). The *AMPD1* gene has the chromosomal locus 1p13-21 and encodes isoenzymes that are highly expressed by skeletal muscle (Morisaki et al., 1990). Two other forms of AMPD, L and E, are expressed in the liver and red blood cells (erythrocytes), respectively. The gene sequence of *AMPD1* comprises 22,455 base pairs (bp) and 16 exons (Sabina et al., 1990). C34T (rs17602729), which represents the transition C>T at the 34th nucleotide, is a nonsense mutation that results in the formation of a premature stop codon in the second exon; it is the most common type of *AMPD1* polymorphism among Caucasians, with a frequency of 10%-15%. This type of mutation occurs in more than 2% of Caucasian and African-American populations (Morisaki et al., 1992; Verzijl et al., 1998; Toyama et al., 2004; Rubio et al., 2005).

There are two types of AMPD deficiency: transmissible (or hereditary), and extrinsic (or acquired). The former is caused by the homozygous form of the C>T transition and results in reduced enzyme activity (Morisaki et al., 1992). The latter arises from a limitation of AMPD transcript availability, which results in several conditions, particularly in skeletal muscle, e.g., muscular dystrophy, or in inflammatory disorders (Hanisch et al., 2008). *AMPD1* deficiency is one of the main causes of metabolic disorders among Caucasians. There are different types of *AMPD1* deficiency; asymptomatic inherited deficiency constitutes the largest group (Sabina et al., 1990). The effect of *AMPD1* on physical activity and obesity has been

documented (Safranow et al., 2011; Cieszczyk et al., 2012). However, the role of *AMPD1* gene polymorphism in cardiovascular disease (CVD) is still controversial.

More than 20% of people in industrialized countries have high blood pressure (BP) (Binder, 2007), which is known as a potent factor in the development of life-threatening diseases such as stroke, end-stage renal disease, and myocardial infarction (Whitworth and Chalmers, 2003). By definition, essential hypertension (EH) has no identifiable cause and it accounts for more than 95% of all hypertension cases. EH is a polygenic disorder, and several environmental factors, such as obesity, stress, and even eating habits, play a direct role in its etiology (Whelton, 1994). Several studies in both human and animal models have shown that more than two-thirds of BP variation is related to genes (Harrap, 1986; Kurtz and Spence, 1993; Hong et al., 1994). At least 10 polygenes may influence BP regulation (Harrap, 1986).

Malaysia consisted of different sub ethnic groups (Hatin et al., 2014) which 71% of all deaths in 2002 were due to chronic disorders, such as CVD. Based on a World Health Organization (WHO) report for 2010, the average rate of CVD in Malaysia was 2.5%, while the minimum and maximum rates were 1.4% and 25%, respectively (WHO, 2010). CVD is the number one cause of death globally; more people die annually from CVD than from any other cause (Alwan, 2011). Almost 9.4 million deaths each year, or 16.5% of all deaths, can be attributed to high BP (Lim et al., 2012). This includes 51% of deaths due to stroke and 45% of deaths due to coronary heart disease (WHO, 2010).

We hypothesize that the common *AMPD1* gene polymorphism C34T plays a role in hypertension. We genotyped subjects with and without hypertension, and assessed their phenotypic characteristics to compare both groups in terms of the genotype-phenotype relationship.

MATERIAL AND METHODS

Study population

This study comprised 200 hypertensive patients who had no monogenic disorders that caused secondary hypertension, and who regularly took antihypertensive medication (the case group), and 200 normotensive subjects who were eligible to donate blood (the control group).

The subjects belonged to three main Malaysian ethnic groups: Malays, Chinese, and Indians. This association study was carried out with the ethical approval of the National Malaysian Research Registry (NMRR-12-456-12376) [KKM/NIHSEC/08/0804/P12-519] on December 6, 2012. The patients completed an informed consent form before participation in this survey and proper precautions were made to protect the privacy of all subjects. In addition, a questionnaire was distributed to all participants that recorded information on sociodemographic factors, medication status, history of hypertension in immediate relatives, and history of other diseases that could be a cause of secondary hypertension and diabetes. The follow-up protocol was made by visiting patients at the health clinic (Klinik Kesihatan, Seremban, Malaysia). The samples for the control group were obtained from individuals who donated blood in the locality of the clinic from 2011 to 2012. The three different ethnic groups (Malays, Chinese, and Indians) enabled us to compare the allele frequency and other differences between the normotensive and hypertensive groups. They were tested for the C34T polymorphism: TT (or homozygous - a rare mutation); CT (or heterozygous); and CC (common or normal homozygous).

Hypertensive subjects

Two hundred hypertensive subjects comprising both men (54.5%) and women (45.5%) with a mean age of 60.0 ± 11.3 years (odd ratio 51 to 75 years), were studied. The ethnic distribution of the group was Malays 108/200 (54%), Chinese 56/200 (28%), and Indians 36/200 (18%). Out of 200 hypertensive subjects, 105 (52.5%) were diabetic, while the rest 95 (47.5%) were not. There was a familial history of high BP in 112/200 of the subjects (56%), and the remaining 88/200 (44%) had no familial history of hypertension. Overall, 130/200 (65%) of the patients had high systolic blood pressure (SBP) during follow-up, while 50/200 (25%) had high diastolic blood pressure (DBP) at the time of recruitment.

Normotensive subjects

The control group comprised 200 people from three ethnic groups: Malays, 112 (56%), Chinese, 54 (27%), and Indians 34 (17%). The mean age of the controls was 28.6 ± 8.0, (odd ratio 18 to 31 years).

Sample collection and study measurements

Hypertension diagnosis was based on blood pressure measurements conducted at least three times within a 10 min period. Then, the average SBP and DBP were determined, and the arterial blood pressure (ABP) was calculated as [(2 x DBP) + SBP)]/3. The pulse pressure was defined as SBP minus DBP. The body mass index (BMI) was also determined. To eliminate erroneous extreme blood pressure values, all SBP values of >180 or <85 mmHg and all DBP values of >110 or <40 mmHg were used unless the difference between the first and second measurements was >10 mmHg; this phenomenon was more common in some subjects who had taken antihypertensive drugs. Thus, 10 and 5 mmHg were added to adjust SBP and DBP, respectively, as previously recommended (Cui et al., 2003). Blood samples were obtained for biochemical testing and to extract genomic DNA. Vein blood was collected after 8-12 h of fasting and 10 min of rest to determine the lipid profile and fasting blood sugar (FBS). The clotted blood samples were centrifuged, and the serum was quickly frozen in aliquots and kept at -80°C until required for further analysis.

Genotyping and single nucleotide polymorphism (SNP) selection

AMPD1 was selected based on its effect on metabolic syndrome and inflammatory factors. It has been reported that the C34T polymorphism of *AMPD1* may reduce some of the adverse effects of the inflammatory response, such as tumor necrosis factor-alpha (TNF-α) production (Wagner et al., 1998). In addition, reduced physical activity has been observed among mutated carriers of *AMPD1* (Cieszczyk et al., 2012). The gene and its polymorphism were selected from published research found on PubMed.

DNA extraction

Genomic DNA was extracted using a Vacutainer® (BD Diagnostics, Oxford, UK)

containing 5 mL K3-EDTA anticoagulant (EDTA is ethylenediaminetetraacetic acid) and an innuPREP Blood DNA mini kit (Matrioux Snd Bhd, Germany), and stored at -80°C until required. The purity of the extracted DNA for all normotensive and hypertensive samples (400 subjects) was determined by measuring the ratio of the absorbance at 260 and 280 nm ($A_{260}/_{280}$) before proceeding to molecular analysis.

Polymerase chain reaction (PCR) amplification conditions

The second exon of the *AMPD1* gene was amplified by PCR from the genomic DNA of the whole blood. The forward primer was 5'-CAT ACA GCT GAA GAG ACA-3' and the reverse primer was 5'-AAC ACT GCT GAA AAA TAG-3' (Lim et al., 2012). The primers used to amplify the human *AMPD1* gene polymorphism C34T were selected from the published research (Online Mendelian Inheritance in Man, 102770). The PCR was carried out in a total volume of 25 μL which comprised: 5 μL Master Mix 5X (Ampliqon, PCR Enzymes & Bioreagents, Denmark); 17.5 μL sterile dH_2O; 7.5 nmol of each primer (Biosune, China); and 40 ng genomic DNA. The amplification protocol was: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55.4°C for 30 s, and extension at 72°C for 28 s; and a final extension at 72°C for 5 min. The size of the expected PCR product was 119 bp. The amplification was executed using a C1000™ Thermocycler (Bio-Rad, USA). The PCR product quality was checked by electrophoresis on 2% agarose gel stained with GelRed (nucleic acid gel stain, 10,000X in dimethyl sulfoxide, USA), followed by a 20-1000 bp DNA marker, whereupon it was photographed using a GelDoc Imaging System (Bio-Rad, USA).

Restriction fragment length polymorphism (RFLP)

Following PCR, enzymatic digestion was carried out by RFLP to detect the C34T polymorphism in the second exon of *AMPD1* based on the method reported previously (Safranow et al., 2009). The 119-bp PCR product was cleaved into 98 and 21 bp sequences in the presence of the normal fragment. The C>T substitution changes the ACGT fragment of the recognition site to ATGT. The enzyme-incubated PCR products were electrophoresed on 10% polyacrylamide gel and stained with GelRed. The exact size of the restriction product was measured using a DNA marker (20 -1000 bp DNA Ladder; TaKaRa, Japan). Fragments were captured with a GelDoc Imaging System (Bio-Rad, USA).

Statistical analysis

The chi-squared test was used to analyze differences in the *AMPD1* genotype and the distribution of alleles between the case and control groups. The general linear model and one way analysis of variance were applied to compare genotype and phenotype frequencies. The Hardy-Weinberg equilibrium (HWE) was used to check the genotype distributions. The Student *t*-test was used to compare the mean between and within the groups. The statistical tests were performed using the SPSS software (version 20.0, Chicago, IL, USA) and $P < 0.05$ was considered statistically significant.

RESULTS

Demographic and clinical characteristics of study subjects

A comparison between the case and control groups is given in Table 1. Subjects aged 60.06 ± 11.33 (95%CI age range 51-75 years), followed by 28.65 ± 8.03 (95%CI age range 18-31 years) constituted the biggest proportion of samples within the case and control groups, respectively. Incidentally, there were significantly more males than females among the hypertensive subjects (109 (54.5%) compared with 91 (45.5%), respectively), but there were equal numbers of either gender among the normotensive subjects.

Table 1. Sociodemographic and blood biochemical characteristics of the subjects.

Characteristics	EH subjects	Healthy subjects	P value
Number (N)	200	200	-
Gender (M/F)	109/91	100/100	-
Age (years)	60.06 ± 11.33	28.65 ± 8.03	<0.05
FBS (mM)	6.37 ± 2.28	4.41 ± 1.11	<0.05
LDL-C (mM)	3.01 ± 1.06	4.55 ± 1.35	<0.05
HDL-C (mM)	1.29 ± 0.35	1.12 ± 0.30	>0.05
TC (mM)	4.6 ± 1.08	5.34 ± 1.23	>0.05
TG (mM)	1.5 ± 0.58	1.68 ± 1.06	<0.05
SBP (mmHg)	148.66 ± 20.81	125.78 ± 14.82	<0.05
DBP (mmHg)	81.99 ± 11.54	78.81 ± 7.69	<0.05
BMI (kg/m^2)	26.79 ± 4.98	24.76 ± 4.95	>0.05

EH = essential hypertension; FBS = fasting blood sugar; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TC = total cholesterol; TG = triglyceride; SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index; Data presented as means ± SD.

Genotype frequencies

The results of gel electrophoresis for the C34T polymorphism of the *AMPD1* gene are shown in Figure 1. Based on the HWE, there was a significant association between the C34T polymorphism of *AMPD1* and EH in the Malaysian subjects. The allele (P < 0.0001) and genotype (P < 0.001) frequencies within the case and control groups were significantly different (Table 2). The frequency of the C allele in the control group was much greater than in the case group (89.25 *vs* 45.50%, respectively). In contrast, the frequency of the T allele in the case group was much greater than in the control group (54.5 *vs* 10.75%, respectively). No TT genotype was observed in the control group. The frequency of hypertension was 9.94 times higher in the T allele carriers than in the C carriers (OR = 9.94; 95%CI = 6.851-14.434). The majority of normotensive subjects carried the homozygous CC genotype [157 (78.5%)], and the majority of hypertensive subjects carried the heterozygous CT genotype [158 (79%)] (Table 2).

Clinical patterns of different genotypes in the hypertensive group

The clinical features of the 200 hypertensive subjects with the C34T polymorphism of the *AMPD1* gene are given in Table 3. The CC genotype had the lowest frequency in the population (12 subjects) and the lowest level of SBP observable in that group (132.0 ± 16.5,
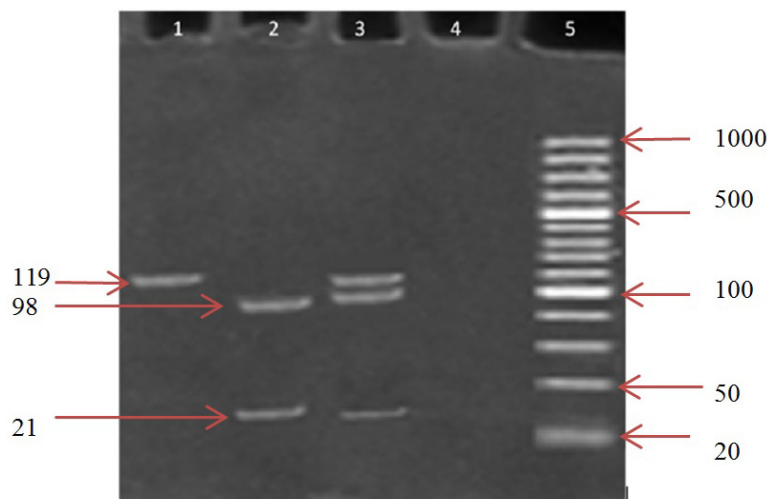
Figure 1. Results of electrophoresis on agarose gel for the C34T polymorphism of the *AMPD1* gene. *Lane 1* = wild type, 119 bp; *lane 2* = homozygous, 98 and 21 bp; *lane 3* = heterozygous, 119, 98, and 21 bp; *lane 4* = negative control; *lane 5* = 20-1000-bp DNA marker.

Table 2. Genotype and allele frequencies of the subjects.

Subjects (N = 200)	Genotype frequencies			Allele frequencies	
	CC	CT	TT	C	T
Hypertensive	12 (6%)	158 (79%)	30 (15%)	182 (45.50)	218 (54.50)
Normotensive	157 (78.5%)	43 (21.5%)	-	357 (89.25)	43 (10.75)
P value	<0.001			<0.0001	

P < 0.05). In contrast, DBP was higher in CC carriers than in CT and TT carriers (88.5 ± 12.7, P < 0.05). Likewise, the lowest pulse pressure was detected in the CC carriers, while the CT carriers had the highest pulse pressure values (43.5 ± 24.8 *vs* 69.4 ± 24.6, P < 0.05). Interestingly, we found for the first time that in subjects that abstained from alcohol, only 4% had the CC genotype, while the frequency of the CT and TT genotypes was significantly higher (results not shown). We did not observe any association between normotensive groups.

Table 3. Clinical features and genotypes of the hypertensive subjects.

	CC (N = 12)	CT (N = 158)	TT (N = 30)	P value
BMI (kg/m^2)	27.0 ± 4.2	26.8 ± 5.0	26.4 ± 5.1	P > 0.05
FBS (mmol/dL)	6.7 ± 2.8	6.4 ± 2.3	5.8 ± 1.5	P > 0.05
TC (mmol/dL)	5.0 ± 1.0	4.5 ± 1.1	4.7 ± 0.9	P > 0.05
HDL-C (mmol/dL)	1.1 ± 0.3	1.2 ± 0.3	1.4 ± 0.4	P > 0.05
LDL-C (mmol/dL)	3.5 ± 1.1	2.9 ± 1.0	2.9 ± 0.9	P > 0.05
Triglycerides (mmol/dL)	1.5 ± 0.6	1.4 ± 0.5	1.5 ± 0.5	P > 0.05
SBP (mmHg)	132.0 ± 16.5	150.4 ± 20.7	146.0 ± 20.0	P = 0.009
DBP (mmHg)	88.5 ± 12.7	81.0 ± 11.4	84.5 ± 10.6	P = 0.03
Pulse pressure (mmHg)	43.5 ± 24.8	69.4 ± 24.6	61.4 ± 23.7	P = 0.02
MAP (mmHg)	103.0 ± 7.9	104.1 ± 9.8	105.0 ± 9.1	P < 0.05

Data are reported as means ± SD or number (percentage) of patients with indicated genotype; BMI = body mass index; FBS = fasting blood sugar; TC = total cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure.

DISCUSSION

To the best of our knowledge, no one has attempted to analyze the association between an *AMPD1* polymorphism and EH in a Malaysian population, and this is the first comprehensive report on the C34T polymorphism of *AMPD1*.

In the present study, the second exon of the *AMPD1* gene was analyzed in hypertensive and normotensive subjects. The same outcomes were reported by Safranow et al. (2009) using the same method (RFLP), with clear evidence from genotyping. Our results revealed that in the Malaysian population, the most common *AMPD1* polymorphism (C34T) is strongly associated with EH (P < 0.001). In other words, a significant association was observed in hypertensive subjects. In contrast, no significant association was observed in the normotensive group. We assumed that C34T may lead to the development of EH and found that C allele carriers have a lower risk of becoming hypertensive, while T allele carriers have a higher risk (OR = 9.49, 95%CI = 5.62-16.02), which may show that T allele carriers do not benefit from the compensatory effect of adenosine on the development of hypertension (Shryock and Belardinelli, 1997), while C allele carriers may experience protection from hypertension.

In this study, we found that the C34T polymorphism of the *AMPD1* gene leads to increased SBP and DBP, while previous studies suggested that *AMPD1* may have promising effects on cardiovascular diseases (Feldman et al., 1999; Loh et al., 1999; Anderson et al., 2000). SBP was significantly lower in the CC carriers, in contrast to the results reported by Safranow et al. (2009). In comparison with Safranow et al.'s study (2009) we detected 30 EH subjects who were carriers of the TT genotype. Moreover, Safranow et al. (2009) reported no significant association between SBP and genotype, although DBP was higher in the CC genotype carriers than in the other subjects. In this study, we found a 2% *AMPD1* deficiency in the CT (or heterozygous) genotype, which agrees with previous studies (Morisaki et al., 1992; Verzijl et al., 1998). However, our findings from this study were not in general agreement with those of previous researchers, who claimed that *AMPD1* polymorphisms have no harmful effect on the human body (Verzijl et al., 1998).

In this study, there was no significant association between genotypes and BMI, which is in agreement with previous studies (Rico-Sanz et al., 2003; Kolek et al., 2005; Agewall and Norman, 2006; Collins et al., 2006). In addition, we did not observe a significant association between genotypes and the level of FBS. Although TT carriers had low levels of FBS, the previous studies have demonstrated that mutation in *AMPD1* may be related to insulin resistance or a reduced level of obesity and hyperglycemia among people who suffer from coronary artery disease (Goodarzi et al., 2005; Safranow et al., 2009). In this study, however, we did not observe any significant association between genotype carriers and type 2 diabetes or obesity in subjects (P > 0.05). Theoretically, AMP-activated protein kinase (AMPK) is influenced by *AMPD1* activity, which induces cellular glucose uptake and affects the incidence of diabetes (Gerbitz et al., 1996). We did not observe any significant difference between the case and control groups with regard to lipid profile (P > 0.05). *AMPD1* is mostly expressed in skeletal muscle; therefore, the reason this gene has an impact on EH remains unclear.

Adenosine, which is released as a consequence of *AMPD1* deficiency, is capable of abating the expression of TNF-α (Wagner et al., 1998). TNF-α is a potent inflammatory factor, so it would be expected to reduce the development of hypertension; the current study shows that 79% of hypertensive subjects are carriers of the CT genotype (Table 2). It has already

been shown that TT carriers have a higher level of TNF-α compared with CC carriers (Mizuno et al., 2006). This may explain why the frequency of hypertension is higher in T carriers than in wild-type carriers. As shown in Table 2, we did not observe any TT carriers in the control group.

Several advantages of adenosine production have been suggested, including reducing hypertension (Leesar et al., 1997; Antman et al., 2000; Pomerantz et al., 2000). However, in this study we found that despite *AMPD1* deficiency, which is observed among heterozygous and mutated homozygous carriers, adenosine has no compensatory effect on the development of hypertension in patients. Cieszczyk et al. (2012) demonstrated that T allele carriers are less athletic than C carriers. Although some studies have shown that the C34T polymorphism of *AMPD1* has an advantageous effect on heart failure survival (Loh et al., 1999), other studies totally disagree with this assumption (Morisaki et al., 1990; Anderson et al., 2000). This may be due to the nature of the local release of adenosine in myocytes or the catalytic activity of *AMPD1*. Therefore, this discovery may help us to learn more about hypertension, as more physical activity has an inverse effect on its development.

Study limitations

The sample size of this study was relatively moderate. The control subjects were younger than the hypertensive subjects owing the randomness of the selections. Another limitation was the measurement *AMPD1* enzyme activity. We did not know the exact *AMPD1* enzyme activity that would have enabled us to determine the relationship between enzyme activity and EH status. Unfortunately, owing to the limited information on the effect of *AMPD1* gene polymorphisms on hypertension, it was very difficult to compare the outcomes of different populations with our findings.

CONCLUSION

In brief, the present study shows that the C34T polymorphism of *AMPD1* is significantly associated with EH. C34T is an independent risk factor for EH and a potential genetic marker for increased susceptibility to EH in Malaysians. Our results could be extended to larger populations because we did include different ethnicities. Our study suggests that *AMPD1* genotypes may provide new insight into the etiology of EH. The C34T polymorphism alone may not be the determining factor for EH, which is a multifactorial disease, but it could improve our understanding of EH and its probable causes. Hypertension is predicted to affect more than 1 billion people by 2025. Although treatment of hypertension is possible, applying and manipulating the results obtained in this study may help prevent this "silent killer".

Conflicts of interest

The authors declare no conflict of interest.

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